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(54) Title: METHOD FOR DETECTING A PARTICULAR PLANT SPECIES IN A PRODUCT

(57) Abstract

A method for detecting a particular plant species in a product such as a foodstuff, said method comprising extracting chloroplast DNA from said product and analysing said DNA to detect a sequence which is characteristic of said particular plant species. Regions of the chloroplast DNA which contain suitable characteristic sequences have been identified. The method may be adapted so that the amount of the detected species within a particular sample is determined. The method can be used to identify adulterant plant species in fruit products and oils and is particularly suitable for identifying mandarin DNA. Novel reagents and particularly oligonucleotides for use in the method are also claimed, as well as diagnostic kits for carrying out the method of the invention.

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METHOD FOR DETECTING A PARTICULAR PLANT SPECIES IN A PRODUCT

The present invention relates to a method for detecting or identifying plants or plant derived material within food products, to reagents and in particular oligonucleotide primers for use in said methods. The methods may be used to detect certain fruit species in particular citrus fruits such as mandarin, for example within fruit containing food products or juice products, or particular oil components such as olive oil, to determine the authenticity of said products.

The sales of processed fruit juices and other food products produced from fruit pulps and purees such as such as preserves (jams, marmalades,)and yoghurts represents a large commercial market and this has led to increased concerns from producers, consumers and regulatory authorities regarding the authenticity of such products. Cases of adulteration of fruit juices with substances such as beet sugar, pulp wash and other juices have been documented.

Such adulteration renders it necessary to carry out authenticity testing on these juices and on fruit pulps and purees used in the production of food products. A still further need for testing arises in the area of nursery stock procurement when verifying the genetic make-up of trees and bushes.

Particular problems vary from state to state depending upon local legislation. For example, in the United States, the products of the citrus hybrid 'Ambersweet' may be sold as orange products, but in Europe, Trading Standards Statutes do not permit this. Major retailers of juice related products thus have a requirement for carrying out authenticity testing, either by themselves or by their suppliers. Furthermore, different fruit products attract different import duties, making source identification necessary by the customs authorities.

The advent of molecular techniques for genetic fingerprinting suggests that this technology may be useful for determining the authenticity of processed f
ruit juices, pulps and purees. In particular, such approaches may be useful in detecting adulteration of fruit juices with other juices and misrepresentation of juices, in terms of both content and country of origin.

One such approach has been illustrated in UK Patent Application No. 2,283,568. In this reference, Randomly Amplified Polymorphic DNA analysis (RAPD) was used to differentiate orange (*C. sinensis*) from other citrus species. However, the specificity of this technique when the citrus fruit is a closely related species such as mandarin (*C. reticulata*) may not be sufficiently good to detect adulteration of orange juices with mandarin juice. Adulteration of orange juice with mandarin juice may be effected for various reasons. For instance, early season oranges may be very light in colour which the addition of mandarin juice may improve, thus making a product more attractive to the consumer.

The situation is similar with respect to many other products which contain plants or plant derived materials. These include oils which may be derived from a wide variety of plant sources. The quality and the cost of oils produced from different plants (olives, sunflowers, oilseed rape etc.) varies a great deal and thus adulteration of oils with cheaper varieties is also a problem.

The applicants have found a method which can distinguish between plant species and in particular can be used to detect the presence of particular plant products in foodstuffs. Surprisingly, it has been found that analysis of chloroplast DNA can be used to distinguish between closely related plant species, including species which are as closely related as mandarin and orange.

The present invention provides a method for detecting a material derived from a particular plant species in a product, such as a food product, said method comprising extracting chloroplast DNA

from said product and analysing said DNA to detect a sequence which is characteristic of said particular plant species.

Preferably the characteristic sequence selected does not exceed 250 base pairs in length. This is due to the degradation of DNA during processing of products for example, in food processing.

Chloroplast DNA can be extracted from products such as fruit juices, concentrates, jams, yoghurts and oils using techniques known in the art. For example, kits are available commercially which will yield DNA from food products such as fruit juices and oils as well as from leaves and fruits of known species which may be required in the methods of the invention for comparison purposes.

In a preferred embodiment, the sequences which are characteristic of the particular plant species are identified using Cleavable Amplifiable Sites Analysis (CAPS). In this technique, amplification primers are prepared based upon known conserved sites which flank variable regions within the DNA. The primers are designed so that in use, the variable region between the conserved sites is amplified. The amplification product may then be analysed.

In a further preferred embodiment, heteroduplex analysis is used in order to detect and even to quantify the amount of particular species or adulterant present. In this technique, amplification primers are selected from conserved chloroplast DNA. Specifically they are selected so that they produce PCR products from different plant species that differ by sequences for example of from 3-10 base pairs which are the result of small insertion/deletion events. Amplification using these primers results in amplification of both the authentic sample and adulterant with equal efficiency. The amplification of authentic samples in the presence of adulterant results in the formation of heteroduplexes The heteroduplexes can be detected and resolved on non-denaturing gels such as polyacrylamide gels (PAGE) and quantified by known fluorimetric methods. The ratio of heteroduplex:homoduplex product allows a simple quantitative measure of adulteration.

Chloroplast DNA is ideally suited to this type of analysis as naturally occurring sequences capable of forming heteroduplexes of this type occur frequently as illustrated hereinafter.

Analysis can be carried out using amplification techniques such as the Polymerase Chain reaction (PCR) or the ligase chain reaction, followed by analysis of the amplified product, for example using restriction fragment length polymorphism (RFLP) analysis, in which the product is digested using restriction enzymes and the restriction fragments are detected on a gel such as an agarose gel using conventional methods. Comparison of the band patterns produced by the product under test with those derived from known plants will provide information as to which plant(s)are present in the product.

Alternatively, sensitive DNA detection techniques using for example labelled oligonucleotide probes, such as radiolabelled probes or fluorescently labelled probes can be used to detect particular sequences as is understood in the art. These methods are particularly useful if, once the variable regions have been identified, they are sequenced and polymorphisms between species identified. These polymorphisms may then be detected in the products under test using methods conventional in the art.

In yet another embodiment, the sequence of the variable region, once identified, can be used to design primers which will amplify a region which is specific to only one plant such as one fruit, for example mandarin.

Particular sequences which are useful in the analysis comprise all or part of the DNA sequence between the chloroplast genes *rbcL* (large subunit of ribulose biphosphate carboxylase) and *psaI* or between tRNA genes *trnT* and *trnL*. In citrus species, these regions are generally of the order of 3.0kb and 1.1kb in length respectively.

Suitable primers for amplification of these particular regions include the following:

rbc L	5'-TAATGAAGGACGTGATCTTGC-3'	(SEQ ID NO 3)
psa I	5'-AGAAGCCATTGCAATTGCCG-3'	(SEQ ID NO 4)

chl A	5'-CATTACAAATGCGATGCTCT-3'	(SEQ ID NO 5)
chl B	5'-TCTACCGATTTCGCCATATC-3'	(SEQ ID NO 6)

SEQ ID NOs 3 and 4 are novel and these primers form a further aspect of the invention. The region between tRNA genes *trnT* and *trnL* and the primers thereof have previously been identified (Taberlet et al., Plant Molecular Biology (1991) 17, 1105-1109).

In particular, the 3.0kb region between the *rbcL* and *psaI* genes are particularly useful as this contains sequences which are characteristic of most fruits, including berries such as raspberries, strawberries blackberries etc., citrus fruits, such as oranges, mandarins, grapefruit and lemons, and others such as apples, pears, plums, bananas etc. This region is long enough to provide characteristic restriction fragment length polymorphisms for all such species.

Particularly useful restriction endonucleases for the analysis of this region are *ApoI* and *Hinf I* or isoschizomers of these enzymes, although others may be employed.

The DNA region between the chloroplast tRNA genes *trnT* and *trnL* has been sequenced in orange (SEQ ID NO 1) and mandarin (SEQ ID NO 2) as illustrated hereinafter (Figure 1), and the polymorphisms identified can be used to distinguish between these very similar fruit species.

Thus in a preferred embodiment, the invention provides a method for detecting the presence of *C. reticulata* in a sample containing *C sinensis* which method comprises extracting DNA from said sample, carrying out an amplification reaction using

primers which are able to amplify a region of DNA comprising part of the *C. reticulata* sequence shown in Figure 1 but not the corresponding part of the *C.sinesis* sequence shown in Figure 1, and detecting the presence of amplified product.

Polymorphisms identified in Figure 1 which can be used in primer design are designated 1 and 2. Specific primers for use in this embodiment include the following:

A	*chl A*	5'-CATTACAAATGCGATGCTCT-3'	(SEQ ID NO 5)
	machl	5'-GAATTTCGAAAATGGATAATGAT-3'	(SEQ ID NO 7)
B	*chl B*	5'-TCTACCGATTTCGCCATATC-3'	(SEQ ID NO 6)
	mbchl	5'-GATTAGAATGAGACATTTCTCCA-3'	(SEQ ID NO 8)
C	*mb2chl*	5'-GAATGAAAACAAAAATGGAGAAA-3'	(SEQ ID NO 9)
	ma2chl	5'-TATATTCGATTCTTATTATCATTA-3'	(SEQ ID NO 10)

SEQ ID NOs 7, 8,9 and 10 are novel and form a further aspect of the invention.

The PCR reaction of the invention may be carried out as part of a multiplex PCR reaction, for instance using primers specific for a conserved region to provide an internal standard. In the case of mandarin determination, a suitable internal standard is derived from the large subunit of the ribulose biphosphate carboxylase (rbc L) gene. Particular primers to achieve this include the following:

rbc L	*rbc L*	5'-GGATTYAAAGCYGGTGTTAAAG-3'	(SEQ ID NO 11)
	rbc L	5'-GATTCGCAGATCCTCYAGAC-3'	(SEQ ID NO 12)

Using the method of the invention, it has proved possible to detect 0.1% mandarin DNA in mandarin/orange DNA mixes, corresponding to the detection of 5pg of mandarin DNA. This analysis has been successfully applied to commercially available concentrate derived fruit juices.

Heteroduplex analysis as outlined above and illustrated below is a further possibility for allowing quantitative determination of adulterant in products. Amplification across an indel sequence

such as indel 1 or indel 2 as illustrated in Figure 1 would produce a suitable detectable heteroduplex product. Specific primers for example for amplication of the indel 2 region of Figure 1 are as follows:

B_{FOR} 5′ AGAAAGATACAATCCCGCTAAACG 3′ (SEQ ID NO 13)

B_{REV} 5′ GTATCCGCAATTCAATATATAGATGGA 3′ (SEQ ID NO 14)

These primers are also novel and form a further aspect of the invention.

The application of this technique to the quantitative detection of adulteration of plant containing products can be used where closely related species or varieties are contained within the product.

When using PCR amplification, the PCR conditions are chosen so as to provide adequate specificity of the reaction. These conditions will be known to the skilled person or could be readily determinable using routine methods. For instance, the reaction is suitably carried out in the presence of magnesium ions, (although the concentration of these ions has not been found to be particularly critical) and annealing temperatures in the range of 50-65°, preferably from 55-60°C depending upon the particular reaction being effected are employed. Reaction times can be varied, for instance between 15 seconds and 1 minute in the cycling, with longer reaction times of from 3 to 10 minutes preferred at the intial denaturation and final polymerisation steps. Examples of suitable conditions include the following steps:

(a) initial denaturation at 94°C for 3 minutes;

(b) 35 cycles of (i) 94°C for 30 seconds;
(ii) 60°C for 15 seconds; and
(iii) 72°C for 30 seconds.

(c) a final stage of (i) 94°C for 30 seconds;
(ii) 60°C for 15 seconds; and
(iii) 72°C for ten minutes
(iv) hold at 18°C.

These conditions are highly specific for mandarin detection when carrying out amplification reactions using primers of SEQ ID NOs 7 and 8. Similar conditions may be employed with primers of SEQ ID NOs 11 and 12, and the results retain good specificity for mandarin although the yield of product may be lower. Where SEQ ID NOs 9 and 10 are employed, the annealing temperature is suitably lower to retain mandarin specificity.

These reactions may be effected in thin walled PCR tubes to allow for rapid and efficient changes in temperature between the various stages.

There may be circumstances, for example, in food inspection, where it is necessary to know the amount of the detected plant species in the sample. For this purpose, the methods of the invention may be suitably adapted to quantify the detected plant species, using technology known to the skilled person. For example, when the method of the invention involves the use of a PCR amplification technique, quantification may be effected using a fluorescence based PCR product quantification system such as the TAQMAN ™ from Perkin-Elmer. This system uses selective cleavage of a probe oligonucleotide during the PCR in order to provide a "real-time" picture of the progress of the PCR reaction. It offers the potential for accurate quantification of PCR products.

A further system by which an amplification reaction such as a PCR reaction may be quantified using fluorescence is described in WO93/06241. In this system, an oligonucleotide which hybridises with the target sequence is immobilised on a waveguide of an evanescent wave detector, and a fluorescent label is provided which becomes bound to the hybridised product but not to the unhybridised immobilised oligonucleotide. The levels of fluorescence generated at each cycle of the amplification reaction can be measured and related to the amount of target sequence present in the sample.

The methods of the invention provide a simple and robust method for determining the authenticity of plant products at the genus and species level. The sensitivity of chloroplast based PCR

reactions in particular is approximately 100-fold greater than that of reactions based on genomic DNA. This provides a much greater level of sensitivity when applied to food products, particularly processed food products. The methods may also be of use for phylogenetic analysis by plant breeders for example in fruit production, particularly by citrus fruit growers, for reliably determining the parentage of any particular line. For instance, the reaction using primer set A above has been used to demonstrate that chloroplast DNA is maternally inherited in citrus species.

Kits for use in the above described method form a further aspect of the invention. Such kits will comprise at least some of the reagents necessary for carrying out the method, in particular, oligonucleotides which are suitable for amplification of a sequence which is characteristic of the particular plant species being detected, such as PCR primers. They may additionally comprise other reagents used in the amplification process such as enzymes like thermostable polymerase enzymes and/or buffers. In some cases, they may also comprise reagents used in the extraction of DNA from food products.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 shows the partial sequences of orange and mandarin DNA amplified using PCR primers chl A and chl B;

Figure 2 shows the results of the application of CAPS analysis to Mandarin/Orange DNA mixes; in this Figure, Lanes 1 and 11 are ØX174 Hae III size markers, Lane 2 is 100% mandarin, Lane 3 is 75% mandarin, Lane 4 is 50% mandarin, Lane 5 is 25% mandarin, Lane 6 is 10% mandarin, Lane 7 is 5% mandarin, Lane 8 is 1% mandarin, Lane 9 is 0.5% mandarin and Lane 10 is 0% mandarin (100% orange);

Figure 3 illustrates PCR assays for mandarin (C. reticulata);

Figure 4 shows the PCR detection of mandarin DNA in mandarin/orange DNA mixes;

Figure 5 shows the results of multiplex PCR analysis of concentrate derived fruit juices including commercially available fruit juices; in this Figure, Lanes 1 and 9 are ØX174 Hae III size markers, Lanes 2, 3 and 4 are concentrate derived orange juices obtained from retail outlets, Lanes 5, 6 and 7 are concentrate derived orange juices prepared from orange juice concentrates, and Lane 8 is concentrate derived mandarin juice prepared from mandarin concentrate;

Figure 6 is the results of heteroduplex analysis using PCR, in this Figure, Lanes 1 and 10 are *ØX174 Hae III* size markers, Lane 2 is 100% mandarin juice, Lane 3 is 50% mandarin juice, Lane 4 is 25% mandarin juice, Lane 5 is 15% mandarin juice, Lane 6 is 10% mandarin, Lane 7 is 5% mandarin juice, Lane 8 is 1% mandarin juice and Lane 9 is 0% mandarin juice (100% orange juice).

Example 1

CAPS Analysis using PCR reactions

DNA was extracted from leaves, fruits and fruit juices using a commercially available kit (Nucleon Phytopure, Scotlab, U.K.). The species from which DNA were extracted are listed below in Table 1

Table 1

Citrus Species		Variety	Origin
C. sinensis	orange	Washington	Spain
C. sinensis	orange	Cadneera	Florida USA
C. sinensis	orange	Valencia	Israel
C. sinensis	orange	Washington	Florida USA
C. sinensis	orange	Valencia	Cyprus
C. sinensis	orange	Washington	Spain
C. sinensis	orange	Pera	Florida USA
C. reticulata	mandarin	Clementine	Italy
C. reticulata	mandarin	Fortune	Cyprus
C. reticulata	mandarin	Nova	Cyprus
C. limon	Lemon	Verna	Spain
C. limon	Lemon	Femminello	Italy
C. limon	Lemon	Monachello	Italy
C. paradisi	Grapefruit	Red Blush	Spain
C. paradisi	Grapefruit	Marsh	Cyprus
C. paradisi	Grapefruit	Sweetie	Israel
C. unshui	Satsuma	Satsuma	Spain
g x p	hybrid	Melogold	California USA
s x u	hybrid	Ortanique	Cyprus
p x r	hybrid	Minneola	Israel
r x u	hybrid	Kara	California USA
p x r x s	hybrid	Ambersweet	

Abbreviations : g = C. grandis; p = C. paradisi; r = C. reticulata; s = C. sinensis; u = C. unshui.

PCR amplification reactions were performed in a total volume of 50 µl in 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25 °C), 1% Triton X-100, 2.0 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, and dTTP; 125 µM of each primer, and 0.5 units of Taq DNA polymerase . Template DNA was 2.0 µl extracted DNA . Negative controls were autoclaved H_2O. Reactions used 'hot start' PCR for enhanced specificity. This technique, which applies stringent PCR conditions, is well known in the art. In the present case, it was effected by s parating primers from other reagents in a tube by a layer of wax which melts at the primer annealing

temperature (e.g. 55°C). The wax melts during the heating process allowing the primers to hybridise with the target sequence only at a temperature at which they anneal to the target sequence, thus eliminating much non-specific binding which may occur at lower temperatures encountered during the heating process.

Alternatively or additionally, the PCR reaction may be carried out using polymerase enzymes which are active only at high temperatures, for example at 95°C, such as Amplitaq Gold (available from Perkin Elmer). This delays the start of the extension reaction reducing any non-specific primer binding.

Amplifications were for 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min annealing at 60°C and 1 min extension at 72°C. In a final cycle, the extension was carried on for 10 min at 72°C prior to holding at 18°C. Amplification products (10 µl) were separated by electrophoresis in 1.5 % agarose gels and visualized after staining with ethidium bromide solution.

PCR primer pairs used were SEQ ID NOs 3 and 4 and SEQ ID NOs 5 and 6 given above. The products of these reactions were designated the Region A and Region B products respectively.

PCR products were phenol/chloroform extracted and ethanol precipitated. DNA was resuspended in 30µl TE (10mM Tris pH 8.0, 1mM EDTA), and 5µl used for digestion with restriction endonucleases using conditions recommended by the manufacturer. Restriction fragments were either separated by electrophoresis using 2% high-resolution agarose gels (region B), ('Metaphor' agarose, FNC, Rockland, USA, or conventional 1.5% agarose gels (region A). Restriction fragments were visualised after staining with ethidium bromide.

The results are summarised in Table 2.

Table 2

Enzyme	Recognition sequence	Region B(1100bp) trnT-trnL Total Bands	Bands Different	RegionA(3000bp) rbcL-psaI Total Bands	Bands Different
		orange/ grapefruit/ lemon	mandarin	orange/ grapefruit/ lemon	mandarin
Apo I	PuAATTPy	5	2	7	3
Hinf I	GANTC	7	3	6	3
Alu I	AGCT	2	1	~14*	5
Mse I	TTAA	2	2	9	1
DdeI	CTNAG	3	3	8	0
Hpa II	CCGG	3	0	3	4
Bgl II	AGATCT	1	0	6	3
Hind III	AAGCTT	1	0	4	4
Eco RV	GATATC	1	0	8	4
Sst II	CCGCGG	1	0	2	2

* These bands are distinguishable between orange, grapefruit and lemon species.

The results showed that Regions A and B and particularly Region A could be used to distinguish fruit species using CAPS.

Example 2

Application of CAPS Analysis to Mandarin/Orange DNA mixes

In order to assess the potential application of this method to fruit juice authenticity testing, mixtures of mandarin and orange DNA were subjected to CAPS analysis using the technique described in Example 1 above, with SEQ ID NOs 3 and 4 as the primer pair and *Hinf I* as the restriction enzyme.

The results are shown in Figure 2. It is clear that mandarin specific RFLP fragments are detectable in mixes of 25% mandarin and 75% orange DNA.

Example 3

Mandarin Specific PCR

Using the PCR techniques described in Example 1 above, mandarin specific PCR reactions using the following primer pairs was carried out using mandarin/orange DNA mixes.

A	*chl A*	5'-CATTACAAATGCGATGCTCT-3'	(SEQ ID NO 5)
	machl	5'-GAATTTCGAAAATGGATAATGAT-3'	(SEQ ID NO 7)
B	*chl B*	5'-TCTACCGATTTCGCCATATC-3'	(SEQ ID NO 6)
	mbchl	5'-GATTAGAATGAGACATTTCTCCA-3'	(SEQ ID NO 8)
C	*mb2chl*	5'-GAATGAAAACAAAAATGGAGAAA-3'	(SEQ ID NO 9)
	ma2chl	5'-TATATTCGATTCTTATTATCATTA-3'	(SEQ ID NO 10)

In all cases, the presence of mandarin DNA was detectable using these primers.

Example 4

Multiplex PCR to detect mandarin specific sequences

A multiplex PCR reaction using primer pair A from example 3 as well as primers for the large sub-unit of the highly conserved chloroplast ribulose biphosphate carboxylase (rbc L) gene were included at 75 µM. These primers were as SEQ ID NOs 11 and 12 above. Otherwise, the PCR conditions were the same as those described in Example 1 above.

The results are shown in Figure 4. The upper band visible is the mandarin band obtained by reaction of primers A. In this experiment, this reaction was multiplexed with the *rbc* reaction which acts as an internal positive control and produced the control band. The tracks on the left and right sides of Figure 4 contain size markers.

It is clear that this reaction is capable of detecting 5pg mandarin DNA.

Example 5

Multiplex PCR analysis of Concentrate Derived Fruit Juices

DNA for PCR analysis was extracted from commercially available concentrate derived orange fruit juices, and juices prepared from orange concentrates as well as from mandarin juice prepared from mandarin concentrate using a kit available from Nucleon Phytopure, Scotland, Lanarkshire, Scotland.

DNA may be extracted directly from small quantities of juice (up to 200µl). However, for highly acidic juices such as orange juice, the juice was first neutralised by the addition of 1/10 volume of 2M Tris HCl buffer of pH 8.0. DNA extraction was carried out using a modified version of the protocol supplied with the kit as follows:

Cell lysis was effected using the reagents supplied with the kit and following the manufacturers instructions for this step. The lysed sample was placed on ice for 20 minutes and then extracted with chloroform (500µl). The resulting aqueous phase was then extracted with chloroform (500µl) and Nucleon Phytopure DNA extraction silica suspension (100µl). The mixture was shaken for 10 minutes at room temperature and then centrifuged at 10,000g for 5 minutes. The DNA containing upper layer was then transferred to a new tube using a pasteur pipette.

Cold isopropanol (450µl) was added and the tube gently inverted until DNA precipitated. The tube was then centrifuged at 10,000g for 5 minutes to pellet the DNA which was then washed with 70% ethanol. After further centrifugation at 10,000g for 5 minutes, the supernatant was removed and the remaining DNA pellet dried under reduced pressure for 15 minutes.

The DNA was then resuspended in TE buffer (20-50µl) and 2µl used for PCR.

The multiplex reaction carried out was similar to that described in Example 4 above.

The results are shown in Figure 5. It is clear that the only product to show a mandarin specific band is that derived from mandarin concentrate (lane 8) revealing that none of the other juices were subject to contamination or adulteration with mandarin juice.

Example 6

Quantitative Determination of Mandarin Juice in Orange Juice using PCR and heteroduplex analysis

Mandarin juice, orange juice and various mixtures thereof was subjected to heteroduplex analysis. In order to effect this, PCR was effected with the following primers

B_{FOR} 5' AGAAAGATACAATCCCGCTAAACG 3' (SEQ ID NO 13)

B_{REV} 5' GTATCCGCAATTCAATATATAGATGGA 3' (SEQ ID NO 14)

PCR conditions used were as outlined in Example 1. The amplication product spans indel 2 as shown in Figure 1. The product was subjected to electrophoretic separation using a 10% non-denaturing polyacrylamide gel (acrylamide : Bis-acrylamide = 29:1) with a tris-borate EDTA running buffer and at 350V (constant) 60mA, 30W. The gel was subjected to circulation cooling to ensure that the temperature remained of the order of 20°C although the temperature may be allowed to vary provided it does not rise high enough to cause significant denaturation. Alternative conventional electrophoretic conditions (e.g. Temperature or Non-denaturing Gradient Gel Electrophoresis) can be used.

The results on PAGE gel are illustrated in Figure 6. Heteroduplex bands are clearly visible in most adulterated samples.

Claims

1. A method for detecting a particular plant species in a product, said method comprising extracting chloroplast DNA from said product and analysing said DNA to detect a sequence which is characteristic of said particular plant species.

2. A method according to claim 1 wherein said sequence which is characteristic of said particular plant species is 250 base pairs or less in length.

3. A method according to claim 1 or claim 2 wherein the sequence which is characteristic of the particular plant species is a variable region flanked by conserved sequences.

4. A method according to any one of the preceding claims wherein the said sequence which is characteristic of said particular plant species comprises all or part of a DNA between the chloroplast tRNA genes *trnT* and *trnL*.

5. A method according to any one of claims 1 to 3 wherein the said sequence which is characteristic of said particular plant species comprises all or part of a DNA between the genes *rbcL* and *psaI*.

6. A method according to any one of claims 1 to 3 wherein said characteristic sequence comprises a conserved region which includes a small insert or deletion in the said particular species, and wherein the analysis is effected by amplifying a region of said conserved sequence, and detecting heteroduplex formation.

7. A method according to claim 6 wherein said small insert or deletion is from 3 to 10 base pairs in length.

8. A method according to any one of the preceding claims wherein the said particular plant species comprises a fruit species.

9. A method according to claim 8 wherein the fruit species comprises *C. reticulata*.

10. A method according to any one of the preceding claims wherein the said product comprises a citrus fruit product or an oil.

11. A method according to claim 10 wherein said citrus fruit product comprises an orange product.

12. A method according to any one of claims 1 to 5 wherein the analysis is effected by amplification of characteristic sequence, and detection or restriction fragment length polymorphism assay of amplified material.

13. A method according to claim 6 or claim 12 wherein the amplification is carried out using the polymerase chain reaction (PCR).

14. A nucleic acid having a sequence selected from:

rbc L	5'-TAATGAAGGACGTGATCTTGC-3'	(SEQ ID NO 3)
psa I	5'-AGAAGCCATTGCAATTGCCG-3'	(SEQ ID NO 4)

15. A method for detecting and/or identifying a particular plant species in a material, which method comprises taking DNA from material under test, carrying out an amplification reaction using the nucleotide sequences of claim 10 as primers, digesting amplification products with a restiction enzyme and visualising the fragments on a gel, comparing the band pattern obtained with those obtained from known plant species.

16. A method according to claim 15 wherein the restriction endonucleases for the analysis of this region are *ApoI, Hinf I* or isoschizomers of these enzymes.

17. A method according to any one of the preceding claims wherein the amount of the said plant species in said product is quantitated.

18. A method according to claim 18 wherein the amount of said fruit species is quantitated by measuring the degree of fluorescence from a label.

19. A method for detecting the presence of *C. reticulata* in a sample containing *C sinensis* which method comprises extracting DNA from said sample, carrying out an amplification reaction using primers which are able to amplify a region of DNA comprising part of the *C. reticulata* sequence shown in Figure 1 but not the corresponding part of the *C.sinensis* sequence shown in Figure 1, and detecting the presence amplified product.

20. A method according to claim 19 wherein the primers used in the reaction are selected from:

A	*chl A*	5'-CATTACAAATGCGATGCTCT-3'	(SEQ ID NO 5)
	machl	5'-GAATTTCGAAAATGGATAATGAT-3'	(SEQ ID NO 7)
B	*chl B*	5'-TCTACCGATTTCGCCATATC-3'	(SEQ ID NO 6)
	mbchl	5'-GATTAGAATGAGACATTTCTCCA-3'	(SEQ ID NO 8)
C	*mb2chl*	5'-GAATGAAAACAAAAATGGAGAAA-3'	(SEQ ID NO 9)
	ma2chl	5'-TATATTCGATTCTTATTATCATTA-3'	(SEQ ID NO 10)

21. A nucleic acid having a sequence selected from

	machl	5'-GAATTTCGAAAATGGATAATGAT-3'	(SEQ ID NO 7)
	mbchl	5'-GATTAGAATGAGACATTTCTCCA-3'	(SEQ ID NO 8)
C	*mb2chl*	5'-GAATGAAAACAAAAATGGAGAAA-3'	(SEQ ID NO 9) or
	ma2chl	5'-TATATTCGATTCTTATTATCATTA-3'	(SEQ ID NO 10)

22. A method according to claim 19 or claim 20 wherein additional primers which amplify a conserved sequence are also employed.

23. A method according to claim 22 wherein the said additional primers amplify a region of the large subunit of the ribulose biphosphate carboxylase (rbc L) gene.

24. A method according to claim 22 wherein the said additional primers are :

rbc L	*rbc L*	5'-GGATTYAAAGCYGGTGTTAAAG-3'	(SEQ ID NO 11)
	rbc L	5'-GATTCGCAGATCCTCYAGAC-3'	(SEQ ID NO 12)

25. A nucleic acid having a nucleotide sequence selected from

5'-GGATTYAAAGCYGGTGTTAAAG-3' (SEQ ID NO 11)

5'-GATTCGCAGATCCTCYAGAC-3' (SEQ ID NO 12)

26. A method according to claim 6 or claim 7 wherein primers used in the amplification are

B_{FOR} 5' AGAAAGATACAATCCCGCTAAACG 3' (SEQ ID NO 13)

B_{REV} 5' GTATCCGCAATTCAATATATAGATGGA 3' (SEQ ID NO 14)

27. A nucleic acid having a nucleotide sequence selected from

AGAAAGATACAATCCCGCTAAACG (SEQ ID NO 13)

GTATCCGCAATTCAATATATAGATGGA (SEQ ID NO 14)

28. A kit for carrying out a method according to any one of claims 1 to 13, 15 to 20, 22 to 24 or 26.

29. A kit according to claim 28 which comprises oligonucleotides which are suitable for amplification of a sequence which is characteristic of the particular plant species being detected.

30. A kit according to claim 29 which further comprises enzymes and/or buffers suitable for use in the polymerase chain reaction.

31. A kit according to any one of claims 28 to 30 which further comprises reagents suitable for DNA extraction from food products.

Fig.1.

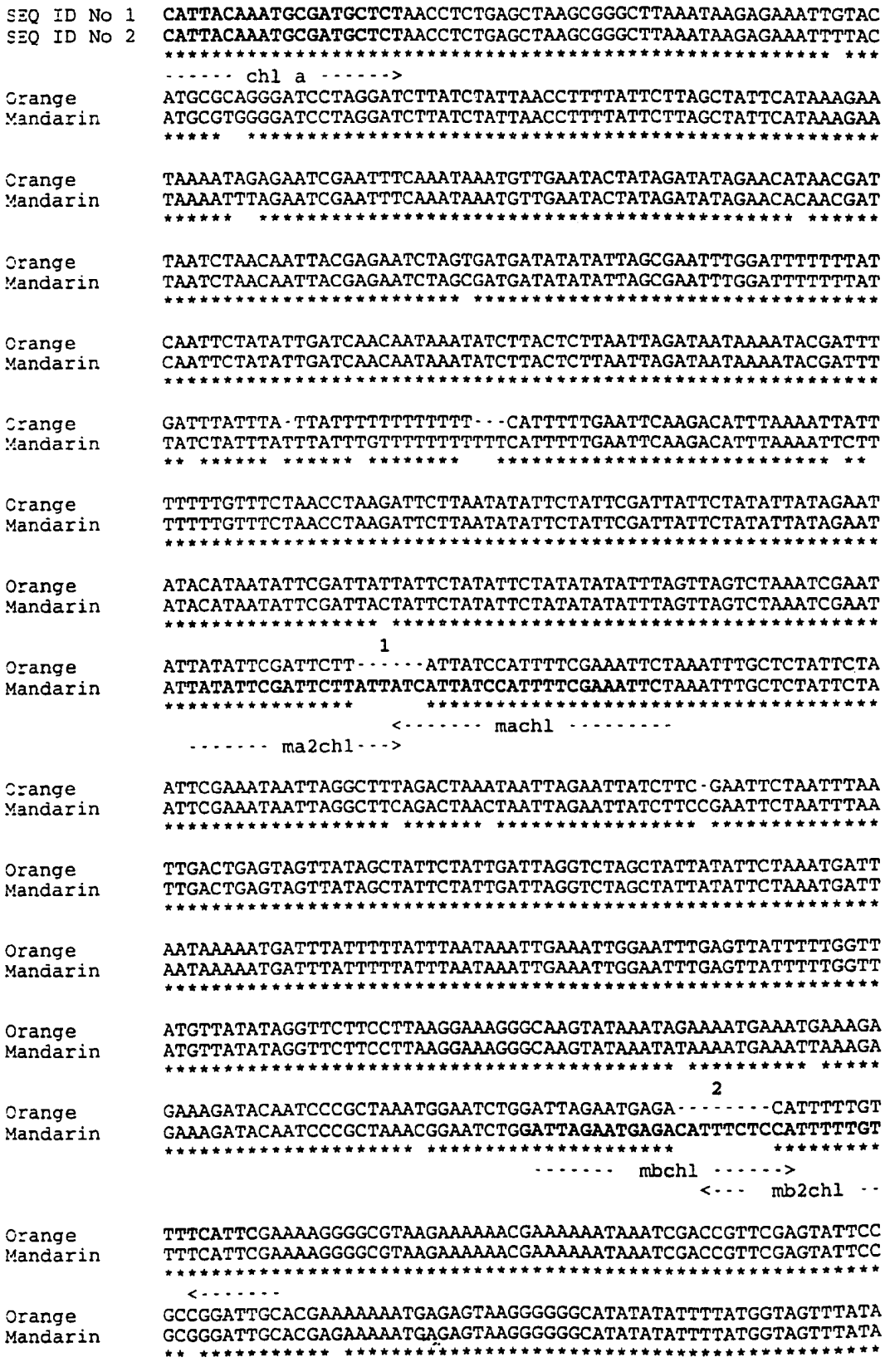

SEQ ID No 1 CATTACAAATGCGATGCTCTAACCTCTGAGCTAAGCGGGCTTAAATAAGAGAAATTGTAC
SEQ ID No 2 CATTACAAATGCGATGCTCTAACCTCTGAGCTAAGCGGGCTTAAATAAGAGAAATTTTAC
------ chl a ------>
Orange ATGCGCAGGGATCCTAGGATCTTATCTATTAACCTTTTATTCTTAGCTATTCATAAAGAA
Mandarin ATGCGTGGGGATCCTAGGATCTTATCTATTAACCTTTTATTCTTAGCTATTCATAAAGAA
Orange TAAAATAGAGAATCGAATTTCAAATAAATGTTGAATACTATAGATATAGAACATAACGAT
Mandarin TAAAATTTAGAATCGAATTTCAAATAAATGTTGAATACTATAGATATAGAACACAACGAT
Orange TAATCTAACAATTACGAGAATCTAGTGATGATATATATTAGCGAATTTGGATTTTTTTAT
Mandarin TAATCTAACAATTACGAGAATCTAGCGATGATATATATTAGCGAATTTGGATTTTTTTAT
Orange CAATTCTATATTGATCAACAATAAATATCTTACTCTTAATTAGATAATAAAATACGATTT
Mandarin CAATTCTATATTGATCAACAATAAATATCTTACTCTTAATTAGATAATAAAATACGATTT
Orange GATTTATTTA-TTATTTTTTTTTTTT---CATTTTTGAATTCAAGACATTTAAAATTATT
Mandarin TATCTATTTATTTATTTGTTTTTTTTTTTTCATTTTTGAATTCAAGACATTTAAAATTCTT
Orange TTTTTGTTTCTAACCTAAGATTCTTAATATATTCTATTCGATTATTCTATATTATAGAAT
Mandarin TTTTTGTTTCTAACCTAAGATTCTTAATATATTCTATTCGATTATTCTATATTATAGAAT
Orange ATACATAATATTCGATTATTATTCTATATTCTATATATATTTAGTTAGTCTAAATCGAAT
Mandarin ATACATAATATTCGATTACTATTCTATATTCTATATATATTTAGTTAGTCTAAATCGAAT
1
Orange ATTATATTCGATTCTT------ATTATCCATTTTCGAAATTCTAAATTTGCTCTATTCTA
Mandarin ATTATATTCGATTCTTATTATCATTATCCATTTTCGAAATTCTAAATTTGCTCTATTCTA
<------- machl ---------
------- ma2chl--->
Orange ATTCGAAATAATTAGGCTTTAGACTAAATAATTAGAATTATCTTC-GAATTCTAATTTAA
Mandarin ATTCGAAATAATTAGGCTTCAGACTAACTAATTAGAATTATCTTCCGAATTCTAATTTAA
Orange TTGACTGAGTAGTTATAGCTATTCTATTGATTAGGTCTAGCTATTATATTCTAAATGATT
Mandarin TTGACTGAGTAGTTATAGCTATTCTATTGATTAGGTCTAGCTATTATATTCTAAATGATT
Orange AATAAAAATGATTTATTTTTATTTAATAAATTGAAATTGGAATTTGAGTTATTTTTGGTT
Mandarin AATAAAAATGATTTATTTTTATTTAATAAATTGAAATTGGAATTTGAGTTATTTTTGGTT
Orange ATGTTATATAGGTTCTTCCTTAAGGAAAGGGCAAGTATAAATAGAAAATGAAATGAAAGA
Mandarin ATGTTATATAGGTTCTTCCTTAAGGAAAGGGCAAGTATAAATATAAAATGAAATTAAAGA
2
Orange GAAAGATACAATCCCGCTAAATGGAATCTGGATTAGAATGAGA--------CATTTTTGT
Mandarin GAAAGATACAATCCCGCTAAACGGAATCTGGATTAGAATGAGACATTTCTCCATTTTTGT
------- mbchl ------>
<--- mb2chl --
Orange TTTCATTCGAAAAGGGGCGTAAGAAAAAACGAAAAAATAAATCGACCGTTCGAGTATTCC
Mandarin TTTCATTCGAAAAGGGGCGTAAGAAAAAACGAAAAAATAAATCGACCGTTCGAGTATTCC
<-------
Orange GCCGGATTGCACGAAAAAAATGAGAGTAAGGGGGGCATATATATTTTATGGTAGTTTATA
Mandarin GCGGGATTGCACGAGAAAAAATGAGAGTAAGGGGGGCATATATATTTTATGGTAGTTTATA

Fig.1 (Cont).

```
Orange      TCCATCTATATTGAATTGCGGATACAGAAAAGATAGAATCATTTCTGACAAGAAAGCAAA
Mandarin    TCCATCTATATTGAATTGCGGATACAGAAAAGATAGAATCATTTCTGACAAGAAAGCAAA
            ************************************************************

Orange      ACACCTTTCGATATATATAAATCCATATCTACGGAATTCTACTGTTTCCGCAGAAATGAA
Mandarin    ACACCTTTCGATATATATAAATCCATATCTACGGAATTCTACTGTTTCCGCAGAAATGAA
            ************************************************************

Orange      AGAAAGGGGGAACAGCATTGAGTTCCTAAAACACAAAGGGGATATGGCGAAATCGGTAGA
Mandarin    AGAAAGGGGGAACAGCATTGAGTTCCTAAAACACAAAGGGGATATGGCGAAATCGGTAGA
            ************************************************************
                                                  <---chl b----------
```

Bases used for mandarin specific primers are shown in bold.

Fig.2.

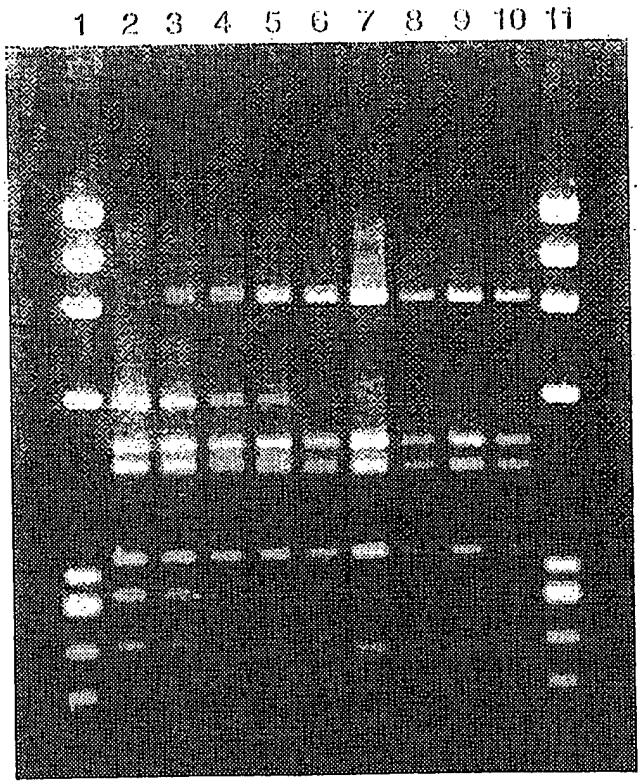


Fig.3.

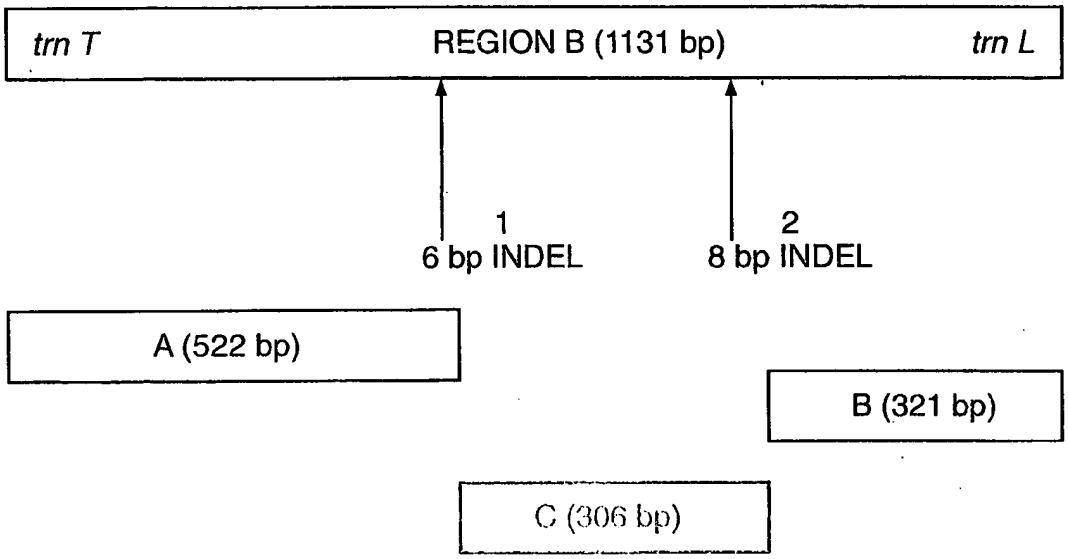

trn T
REGION B (1131 bp)
trn L
1
6 bp INDEL
2
8 bp INDEL
A (522 bp)
B (321 bp)
C (306 bp)

Fig.4.

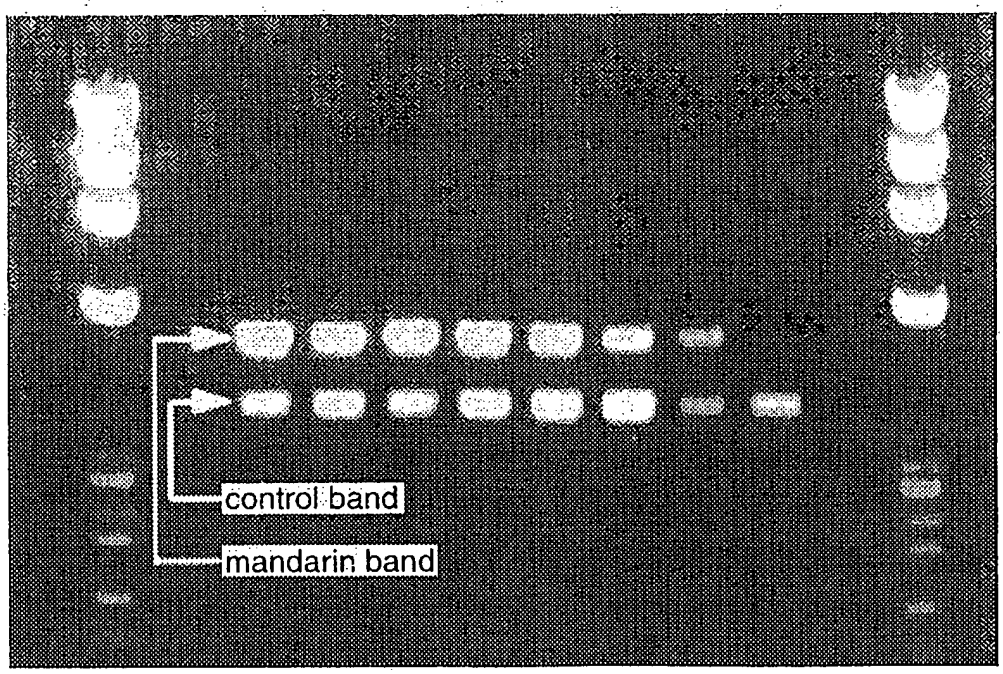
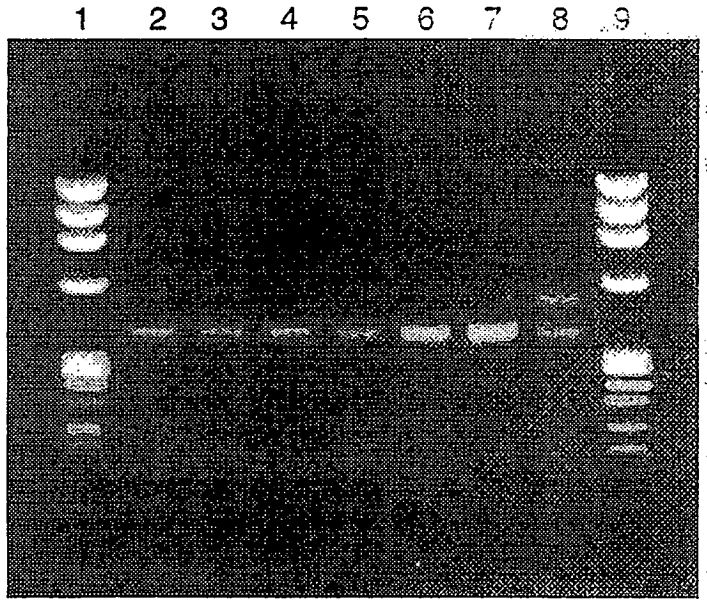

control band
mandarin band
% mandarin DNA
100
50
25
10
5
1
0.1
0

Fig.5.



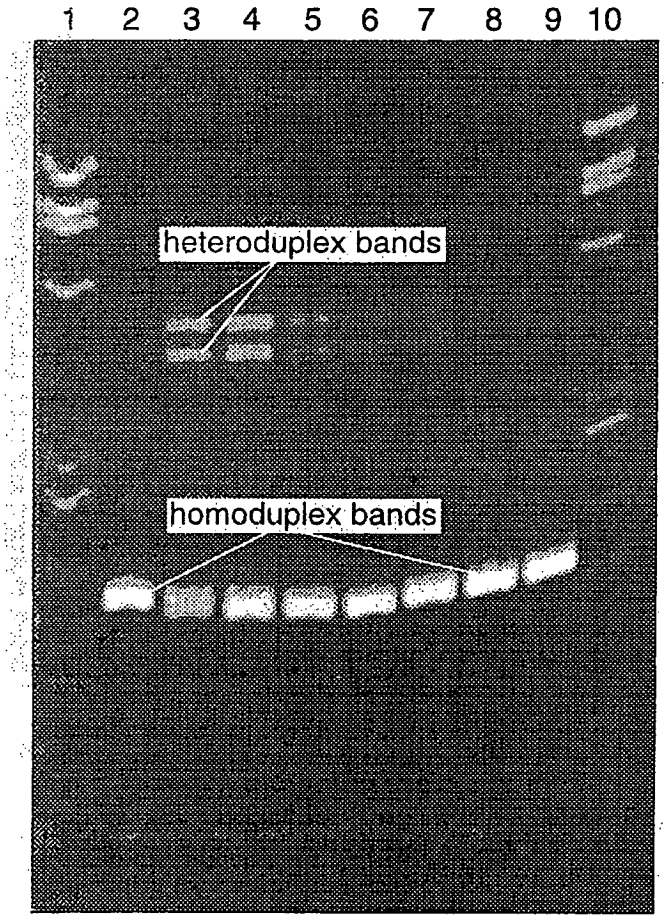
Lanes 1 and 9 øX174 Hae III size markers.

Lanes 2, 3 and 4, concentrate derived orange juices obtained from retail outlets:

Lanes 5, 6 and 7, concentrate derived orange juices prepared from orange juice concentrates: and

Lane 8, concentrate derived mandarin juice prepared from mandarin concentrate.

Fig.6.



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/02688

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 018, no. 334 (C-1216), 24 June 1994 & JP 06 078770 A (KIRIN BIBARETSUJI KK), 22 March 1994, see abstract ---	1-31
X	PATENT ABSTRACTS OF JAPAN vol. 095, no. 006, 31 July 1995 & JP 07 059577 A (KIRIN BIBARETSUJI KK), 7 March 1995, see abstract --- -/--	1-13, 15-19, 28-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
13 January 1998	26/01/1998

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	TABERLET P. ET AL.,: "Universal primers for amplification of three non-coding regions of chloroplast DNA" PLANT MOLECULAR BIOLOGY, vol. 17, - 1991 pages 1105-1109, XP002051822 cited in the application	1-4
Y	see the whole document	8-13,19, 28-31
Y	GB 2 283 568 A (MINI AGRICULTURE & FISHERIES) 10 May 1995 cited in the application see the whole document	8-13,19, 28-31
A	LURO F. ET AL.,: "DNA amplified fingerprinting, a useful tool for determinantion of genetic origin and diversity analysis in Citrus" HORTSCIENCE, vol. 30, no. 5, - August 1995 pages 1063-1067, XP002051823 see whole document esp. discussion	1-13, 15-19, 28-31

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 97/02688

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2283568 A	10-05-95	FR 2711143 A	21-04-95